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Human endoplasmic reticulum oxidoreductin 1-α is a novel predictor for poor prognosis of breast cancer

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Human endoplasmic reticulum oxidoreductin 1-α (hERO1-α) is an oxidizing enzyme that exists in the endoplasmic reticulum and its expression is augmented under hypoxia. It regulates a redox state of various kinds of protein through reoxidation of "client" protein disulfide isomerase. Interestingly, although the expression of hERO1-α in normal tissues was comparatively limited, various types of cancer cells expressed it in large amounts. Therefore, we examined the role of ERO1-α in tumor growth using murine breast cancer line 4T1 and found that knockdown of murine ERO1-α inhibited *in vivo* tumor growth and decreased lung metastasis compared with wild-type 4T1. Moreover, we investigated the relationship between expression of hERO1-α and prognosis in breast cancer patients. Seventy-one patients with breast cancer who underwent surgery between 2005 and 2006 in Sapporo Medical University Hospital (Sapporo, Japan) were analyzed in this study. Significant differences were found between the hERO1-α-positive group (n = 33) and hERO1-α-negative group (n = 38) in nuclear grade ($P < 0.001$) and intrinsic subtype ($P = 0.021$) in univariate analysis. More importantly, in multivariate analysis of disease-free survival by Cox regression, expression of hERO1-α was the only independent prognosis factor ($P = 0.035$). Finally, in univariate survival analysis, patients positive for hERO1-α had significantly shorter disease-free survival and overall survival than those patients negative for hERO1-α. These findings indicate that the expression of hERO1-α in cancer cells is associated with poorer prognosis and thus can be a prognostic factor for patients with breast cancer. (*Cancer Sci* 2013; 104: 1091–1096)

Hypoxia is a physiologically important characteristic that is present in all tumors. Importantly, tumor hypoxia exerts a pronounced effect on malignant progression and metastatic spread of human cancers.[1] Numerous clinical studies have shown that tumor hypoxia predicts decreased local control, increased distant metastases, and decreased overall survival in a variety of human tumors. Hypoxia has been shown to select tumors with an augmented malignant phenotype and increase the metastatic potential of tumor cells.[1,2] It is known that hypoxic cells are relatively resistant to killing by radiation.[3] In addition, because hypoxic cells are non-proliferating and relatively isolated from the blood supply, chemotherapies that target rapidly dividing cells may be less effective for this population of cells and the delivery of chemotherapy to these areas may be compromised. Thus, tumor hypoxia correlates with a more aggressive disease course and limits the effectiveness of anticancer therapy.[4] Human endoplasmic reticulum oxidoreductin 1-α (hERO1-α) is a hypoxia-inducible endoplasmic reticulum-resident oxidase that regulates the post-translational branch of oxidized protein folding.[5,6] It has been identified as a reoxidizer of protein disulfide isomerase (PDI),[7,8] which functions as a disulfide-introducing enzyme for secretory and cell-surface molecules in the cell.[9] Therefore, PDI exists mainly as an oxidized form within a cell. Human ERO1-α is expressed in normoxic cells at very low level. However, it has been shown that hERO1-α is induced in hypoxic cells in response to low oxygen availability.[6] Importantly, the expression level of PDI was also shown to be augmented under hypoxic conditions,[10] suggesting that hERO1-α plays a key role in hypoxic cancer cells in concert with PDI. Although it has been shown that hERO1-α plays a pivotal role in hypoxia-inducible factor 1-mediated vascular endothelial growth factor (VEGF) production,[11] the role of hERO1-α in *in vivo* tumor growth has yet to be elucidated. Many genes associated with breast cancer metastasis have been reported to be upregulated under hypoxic conditions, and hypoxic gene signatures are associated with poor outcome in breast cancer.[12,13]

In this study, we investigated the effect of hERO1-α on tumor progression using the murine ERO1-α-positive metastatic murine breast cancer cell line 4T1. We also investigated the effects of knocking down murine ERO1-α with siRNA. We further investigated the expression of hERO1-α and its clinical relevance in 71 breast cancer patients with long-term follow-up by immunohistochemistry. The expression of estrogen receptor (ER) and nuclear grade were also investigated. We found that a high expression level of hERO1-α in breast cancer tissues was associated with nuclear grade status and was an independent prognostic factor for breast cancer patients after surgery. Thus, understanding the relationships between hypoxia, hERO1-α expression, and tumor growth is crucial for improving current breast cancer therapies.

Materials and Methods

Cell and cell culture under hypoxic conditions. Murine breast cancer cell line 4T1 was purchased from ATCC (Manassas, VA, USA). Cells were cultured in RPMI-1640 (Nissui, Tokyo, Japan) supplemented with 10% FCS. Short hairpin RNA for murine ERO1-α (TR502816) was purchased from OriGene (Rockville, MD, USA) and transfected to 4T1 cells using Lipofectamine RNAiMAX (Life Technologies, Carlsbad, CA, USA). The 4T1 cells were cultured under hypoxic conditions (1% O_2, 5% CO_2, and 94% N_2) for at least 3 days. Cells maintained under normoxic conditions (20% O_2, 5% CO_2, and 75% N_2) were used as controls.

Reverse transcription–PCR analysis. Total RNA was isolated from cultured cells, breast cancer tissues, and normal breast tissues using Isogen reagent (Nippon Gene, Tokyo, Japan). The cDNA mixture was synthesized from 1 μg total RNA by reverse transcription using Superscript III and oligo (dT) primer (Life Technologies) according to the manufacturer's protocol. The PCR amplification was carried out in 50 μL PCR mixture containing 1 μL cDNA mixture, KOD Plus DNA polymerase

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(Toyobo, Osaka, Japan), and 50 pmol primers. The PCR mixture was initially incubated at 92°C for 2 min, followed by 30 cycles of denaturation at 92°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 1 min. Primer pairs used for RT-PCR analysis were 5′-GCCCGTTTTATGCTTGATGT-3′ and 5′-AACTGGGTATGGTGGCAGAC-3′ for human *ERO1-α*. As an internal control *G3PDH* was detected by using the forward primer 5′-ACCACAGTCCATGCCATCAC-3′ and the reverse primer 5′-TCCACCACCCTGTTGCTGTA-3′.

Western blot analysis. Cultured cells were washed in ice-cold PBS, lysed by incubation on ice in a lysis buffer (50 mmol/L Tris-HCl [pH 8.0], 150 mmol/L NaCl, 1% NP40, protease inhibitor cocktail; Complete [Roche Diagnostics, Basel, Switzerland]), and cleared by centrifugation at 21880*g* for 20 min at 4°C. For blockade of free thiols, cells were pretreated for 5 min with 10 mM methyl methanethiosulfonate (Pierce, Rockford, IL, USA) in PBS. Cells were lysed in 1% NP40 in TBS buffer containing 25 mM HEPES, 100 mM NaCl, 10 mM $CaCl_2$, and 5 mM $MgCl_2$ (pH 7.6) supplemented with a protease inhibitor and 5 mM methyl methanethiosulfonate. Post-nuclear supernatants were divided and heated for 5 min at 95°C in non-reducing or reducing SDS sample buffer, resolved by 10% SDS-PAGE, and electrophoretically transferred to PVDF membranes (Immobilon-P; Millipore, Billerica, MA, USA). The membranes were incubated with blocking buffer (5% non-fat dried milk in PBS) for 1 h at room temperature then incubated for 60 min with anti-ERO1-α mAb (Abnova, Taipei, Taiwan), anti-PDI polyclonal antibody (Enzo Life Sciences, Farmingdale, NY, USA) or mouse anti-β-actin mAb AC-15 (Sigma-Aldrich, St. Louis, MO, USA). After washing three times with wash buffer (0.1% Tween-20 in PBS), the membranes were reacted with peroxidase-labeled goat anti-rabbit IgG antibody (KPL, Gaithersburg, MD, USA) for 2 h. Finally, the signal was visualized using an ECL detection system (Amersham Life Science, Arlington Heights, IL, USA) according to the manufacturer's protocol.

Proliferation assay. A cell proliferation assay based on cleavage of the tetrazolium salt WST-1 (DOJINDO, Kumamoto, Japan) by mitochondrial dehydrogenases in viable cells was carried out according to the manufacturer's instructions. The cells were seeded on 96-well microtiter tissue culture plates in 10% serum at a density of 1×10^3 cells/well. After incubation at 37°C for 24–72 h, WST-1 reagent (10 μL) was added to the cells and the cells were incubated at 37°C in 96-well microtiter tissue culture plates for 2 h at 37°C. The amount of formazan dye produced, which directly correlates to the number of metabolically active cells in culture, was quantified by measuring the absorbance at a wavelength of 450 nm using a microtiter plate (ELISA) reader.

***In vivo* studies.** Female BALB/c mice, 5–6 weeks old, were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and used at 6 weeks of age. Mice were maintained in a specific pathogen-free mouse facility at Sapporo Medical University (Sapporo, Japan) according to institutional guidelines for animal use and care. For tumor formation studies, mice were injected with 1×10^5 4T1 or ERO1-α knockdown cells into right mammary glands. Tumor length and width were measured with a caliper. All of the experiments were carried out with five mice /group. Average tumor diameters on day 42 were statistically analyzed using the Mann–Whitney *U*-test. At day 42, numbers of lung metastases were counted and compared.

Enzyme-linked immunosorbent assay. The 4T1 cells were plated at 1×10^4 cells/well in flat-bottomed, 96-well plates for 24 h. Supernatants were diluted and tested for mouse VEGF-A (R&D Systems, Minneapolis, MN, USA) using a sandwich ELISA kit. Absorbance was determined at 450 nm.

Patients and immunohistochemical variables for specimens. Tissue samples were obtained from 71 patients diagnosed with breast cancer in 2005 at Sapporo Medical University Hospital. A total of 71 specimens of primary invasive carcinoma were obtained from resected tumors. All of the specimens used in this study were fixed in neutral 10% buffered formaldehyde, embedded in paraffin, and cut into 5-μm slices. Other background data for the patients are shown in Table 1. The expression of estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor type 2 (HER2), and hERO1-α was determined immunohistochemically in paraffin-embedded tissue specimens. Monoclonal Abs against ER, PgR, and HER2 were purchased from DAKO Japan (Tokyo, Japan). The expression of ER or PgR was designated as positive when at least 10% of the tumor nuclei showed positive staining. The expression of HER2 was classified according to the Hercep Test assay's scoring system, which includes four categories, 0, 1+, 2+ and 3+, based on the intensity and proportion of membrane staining in tumor cells. Positivity was defined as a HER2 score of 3+ for immunostaining or >2-fold increase in HER2 gene amplification, as determined by FISH. The expression of hERO1-α was designated as positive when at least 30% of perinuclear staining within tumor cells was observed. Histological examinations were carried out on slides with paraffin-embedded samples stained by H&E according to the criteria of the Japanese Breast Cancer Society, which are based on the International Union against Cancer TMN classification criteria.

Statistical analysis. The χ^2-test and unpaired *t*-test were used for analysis of two unpaired samples. Disease-free survival and overall survival rates after surgical resection were calculated by the Kaplan–Meier method, and differences in survival curves were assessed by the log–rank test. The Cox proportional hazards model was used for multivariate analysis. All analyses were carried out with SPSS version 18.0 (SPSS Inc.,

Table 1. Background data for patients with breast cancer who participated in this study (*n* = 71)

	n	%
Mean age, years (range)	56	(25–82)
Histological type		
Papillotubular	28	39.4
Solid-tubular	7	9.9
Scirrhous	25	35.2
Others	11	15.5
pT		
pT1 (≤2.0 cm)	22	31.0
pT2 (2.0 ≤ 5.0 cm)	43	60.6
pT3 (>5.0 cm)	6	8.4
pN		
pN (–)	47	66.2
pN (+)	24	33.8
ER, PgR, HER2 status		
ER (+) or PgR (+) and HER2 (–)	42	59.1
ER (+) or PgR (+) and HER2 (+)	10	14.1
ER (–) and PgR (–) and HER2 (+)	11	15.5
ER (–) and PgR (–) and HER2 (–)	8	11.3
Nuclear grade (NG)		
NG1	22	31.0
NG2	27	38.0
NG3	22	31.0
hERO1-α		
(+)	33	46.5
(–)	38	53.5

ER, estrogen receptor; HER2, human epidermal growth factor receptor type 2; hERO1-α, human endoplasmic reticulum oxidoreductin 1-α; PgR, progesterone receptor.

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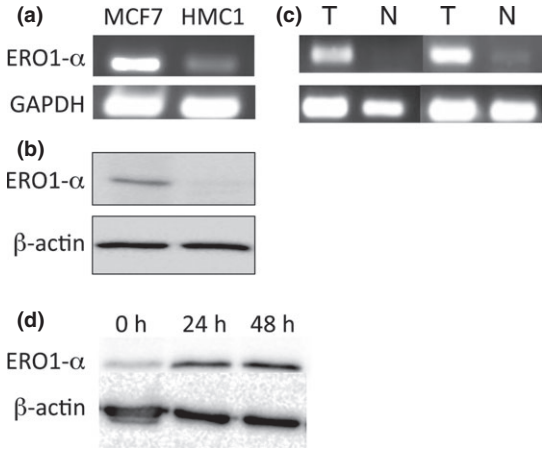

Fig. 1. Expression of human endoplasmic reticulum oxidoreductin 1-α (hERO1-α) in human breast cancer cell lines and tissue samples. (a) Human ERO1-α mRNA levels in MCF7 and HMC1 cells determined by RT-PCR analysis. (b) Western blot analysis of MCF7 and HMC1 cells. (c) mRNA expression of hERO1-α in breast cancer tissues (T) and normal breast tissues (N). (d) Induction of ERO1-α under hypoxic conditions. Western blot analysis of murine breast cancer cell line 4T1 cultured under hypoxic conditions for indicated periods (0, 24 and 48 h).

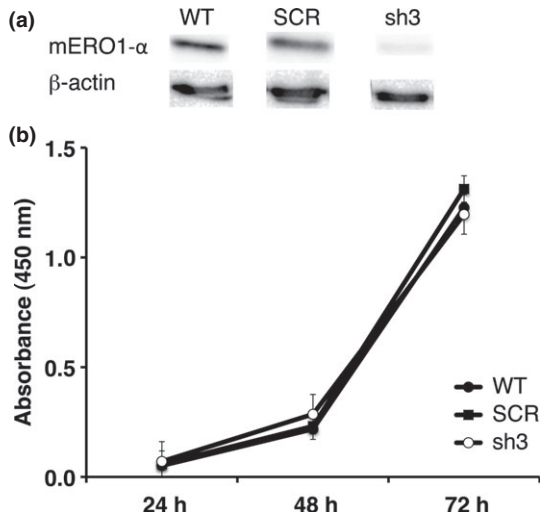

Fig. 2. Functional analysis after endoplasmic reticulum oxidoreductin 1-α (ERO1-α) knockdown in murine breast cancer 4T1 cells. (a) Establishment of a murine ERO1-α (mERO1-α)-depleted 4T1 cell clone by shRNA transfection. ERO1-α was decreased significantly according to Western blot analysis. (b) Cell proliferation was compared by WST-1 assay. Mean ± SD from individual experiments with three replicate assays. Three independent clones were tested and representative data are shown. SCR, 4T1 cell transfected with short hairpin RNA for scramble control; sh3, 4T1 cell clone transfected with shRNA for mERO1-α.

Chicago IL, USA). A *P*-value of less than 0.05 was regarded as statistically significant. All statistical tests were two-sided.

Results

Human ERO1-α expression in breast cancer cell lines and breast cancer tissues. Expression of hERO1-α was detected in the human breast cancer cell line MCF-7 and to a lesser extent in HMC1 cells by RT-PCR (Fig. 1a). Although the expression level of hERO1-α protein was high in MCF7 cells, the protein level of hERO1-α was low in HMC1 cells (Fig. 1b). Interestingly, mRNA of ERO1-α was observed in breast cancer tissues but not in normal mammary gland tissues (Fig. 1c). Notably, the expression of ERO1-α was augmented in 4T1 cells in response to hypoxic conditions, suggesting that tumor hypoxia induces the expression of ERO1-α (Fig. 1d).

Knockdown of ERO1-α by shRNA reduced tumor growth and metastasis. To further examine the functional role of ERO1-α in breast cancer cells, murine breast cancer cell line 4T1 cells were transfected with an shRNA vector targeting murine

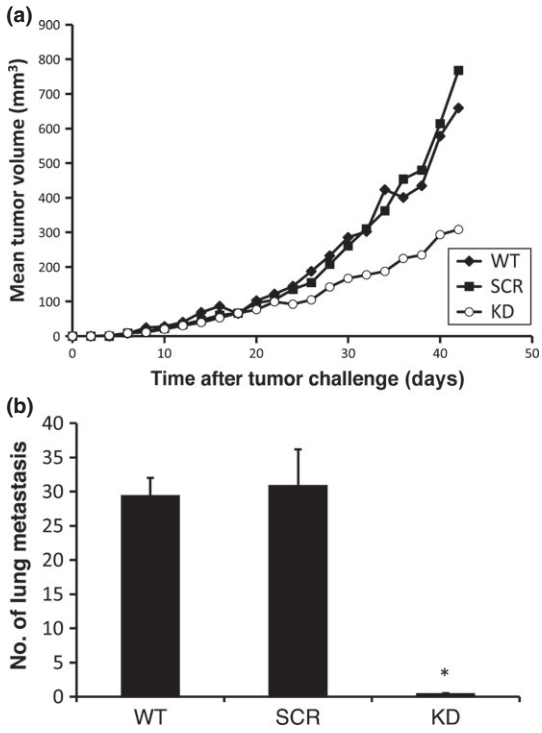

Fig. 3. Expression of endoplasmic reticulum oxidoreductin 1-α (ERO1-α) significantly affects tumor growth and lung metastasis. Female BALB/c mice (five animals/group) were injected with 1×10^5 murine breast cancer 4T1 cells (WT), short hairpin RNA for scramble control (SCR), or ERO1-α knockdown cell (KD) into right mammary glands. At day 42, numbers of lung metastases were counted. Tumor growth (a) and lung metastasis (b) of 4T1 (WT), SCR, and KD were compared. Representative data are shown of four independent experiments. $^*P < 0.005$, paired Student's *t*-test.

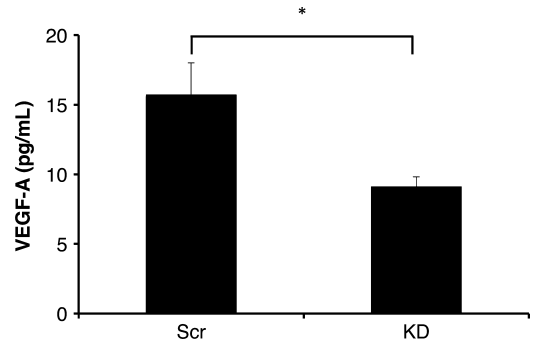

Fig. 4. Endoplasmic reticulum oxidoreductin 1-α (ERO1-α) regulates vascular endothelial growth factor-A (VEGF-A) production from murine breast cancer 4T1 cells. Concentration of VEGF-A in the 24-h culture supernatant from 4T1 cells transfected with short hairpin RNA for scramble control (Scr) or 4T1 cells transfected with short hairpin RNA for mERO1-α (KD) was measured using ELISA. Representative data are shown of three independent experiments. $^*P < 0.01$, paired Student's *t*-test.

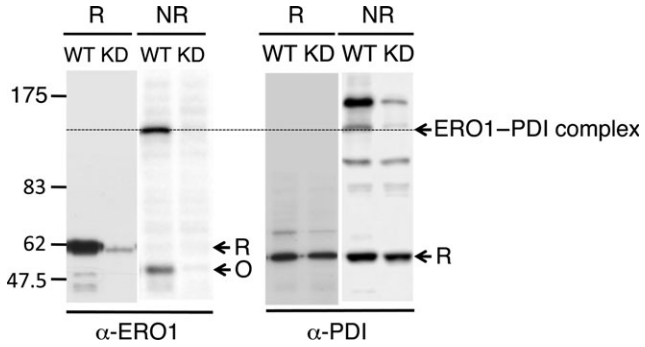

Fig. 5. Endoplasmic reticulum oxidoreductin 1-α (ERO1-α) forms a mixed disulfide linkage with protein disulfide isomerase (PDI) and effects of ERO1-α depletion on the redox status of PDI. Redox status of ERO1-α and PDI in murine breast cancer 4T1 cells (WT) or ERO1-α-depleted cells (KD) was examined by Western blotting under reducing (Red) or non-reducing (NR) conditions. Reduced form (R) or oxidized form (O) of ERO1-α or PDI are indicated. Note that depletion of ERO1-α decreased the ERO1-α–PDI complex.

ERO1-α. In 4T1 cells, successful knockdown of murine ERO1-α expression was confirmed by Western blot analysis (Fig. 2a). The 4T1 cells with downregulation of ERO1-α by shRNA did not show the differences in proliferation assay compared with WT and control shRNA-transfected cells (Fig. 2b). In contrast, knockdown of ERO1-α caused retardation of *in vivo* tumor growth compared with WT 4T1 (Fig. 3a). The number of lung metastases of ERO1-α knockdown 4T1 cells was also significantly decreased compared with that of WT cells (Fig. 3b). These results suggested that the expression of ERO1-α accelerated tumor growth and lung metastasis. To explore the role of ERO1-α in tumor progression and augmented metastasis, we compared VEGF-A production from 4T1 cells transfected with short hairpin RNA for scramble control cells and that from ERO1-α knockdown cells, because VEGF-A is a homodimer whose proper folding through formation of three intramolecular disulfide bonds and two intersubunit disulfide bonds is a prerequisite for its function.[14–16] We found that knockdown of ERO1-α decreased the production of VEGF-A (Fig. 4). These results suggested that tumor hypoxia drove VEGF-A production from tumor through the function of ERO1-α. Thus, ERO1-α plays an important role in tumor growth and metastasis via its upregulation under the condition of tumor hypoxia.

Endoplasmic reticulum oxidoreductin 1-α forms a mixed disulfide linkage with PDI. The formation of native protein disulfide bonds is a critical step in the folding of many secretory and cell-surface proteins. Protein disulfide isomerase serves as a principal catalyst of thiol-disulfide exchange in the lumen of the endoplasmic reticulum. Disulfide transfer to the substrate protein by PDI will result in reduction of the active site of PDI, which must be reoxidized to carry out further oxidation.[7] Mixed disulfide complexes between ERO1-α and PDI have been demonstrated, and these complexes are likely to represent intermediates in the direct oxidation of PDI by ERO1-α *in vivo*.[17] Therefore, we examined whether knockdown of ERO1-α affect the redox status of PDI. Western blot analysis revealed that the ERO1-α–PDI mixed disulfides migrated with an apparent molecular mass of approximately 140 kDa under non-reducing conditions (Fig. 5). Knockdown of ERO1-α in 4T1 cells decreased the ERO1-α–PDI complex, indicating that depletion of ERO1-α decreased the oxidized form of PDI. These data suggested that depletion of ERO1-α decreased disulfide bond formation in VEGF-A, resulting in decreased VEGF-A secretion. Thus, ERO1-α clearly affects the redox status of PDI and production of secreted protein including VEGF-A.

Prognostic factors. Next, we examined whether expression of hERO1-α had an impact on the clinical course of human breast cancer. The expression of hERO1-α was investigated by immunohistochemistry using anti-ERO1-α mAb. Normal breast tissues including mammary ducts revealed negative staining for ERO1-α (Fig. 6a). These results are in good agreement with RT-PCR analysis (Fig. 1c). Thirty-eight cases (53.5%) of the 71 patients with breast cancer showed negative for hERO1-α (Fig. 6b). In contrast, perinuclear staining of

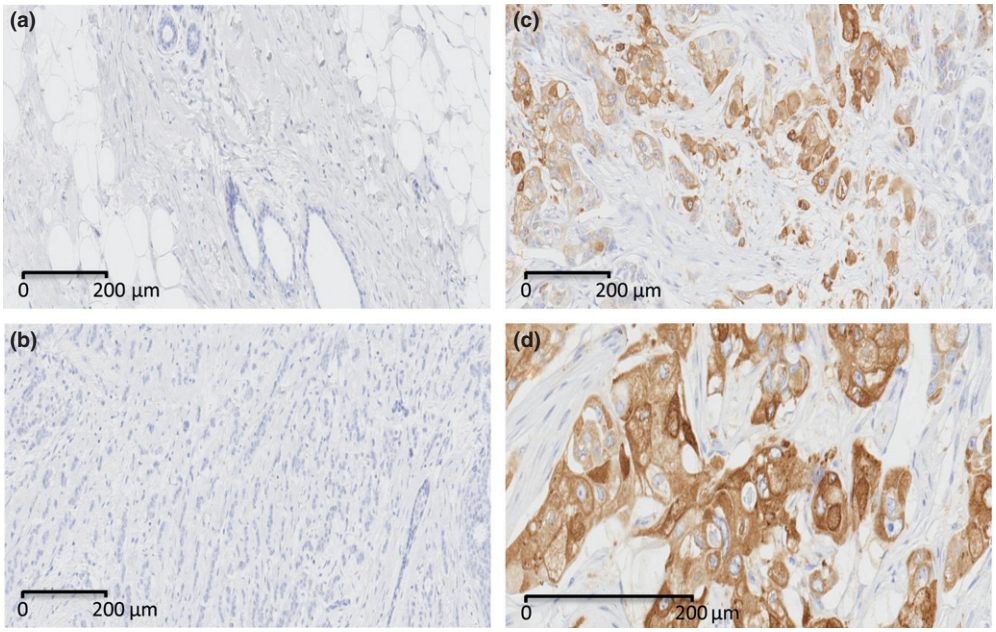

Fig. 6. Expression of human endoplasmic reticulum oxidoreductin 1-α (hERO1-α) in normal breast tissue and breast cancer tissue by immunohistochemical analysis. (a) Normal breast tissue (×200). (b) Breast cancer tissue negative for hERO1-α (×200). (c, d) Perinuclear staining for hERO1-α, indicating endoplasmic reticulum localization in breast cancer cells (c, ×40; d, ×200).

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Table 2. Correlations between human endoplasmic reticulum oxidoreductin 1-α (hERO1-α) status and other clinicopathological factors in patients with breast cancer (n = 71)

	hERO1-α (+)	hERO1-α (−)	*P*-value
Mean age, years (range)	55.4 ± 13.5	58.8 ± 12.6	0.2764
Histological type			
Papillotubular	16	12	
Solid-tubular	4	3	
Scirrhous	9	16	
Others	4	7	
pT			
pT1 (≤2.0 cm)	10	12	0.8376
pT2 (2.0 < 5.0 cm)	19	23	
pT3 (>5.0 cm)	4	3	
pN			
pN (−)	13	11	0.3535
pN (+)	20	27	
ER, PgR, HER2 status			
ER (+) or PgR (+) and HER2 (−)	14	28	0.0210
ER (+) or PgR (+) and HER2 (+)	5	5	
ER (−) and PgR (−) and HER2 (+)	9	2	
ER (−) and PgR (−) and HER2 (−)	5	3	
Nuclear grade (NG)			
NG1	9	13	0.0010
NG2	7	20	
NG3	17	5	
Ly			
Ly (+)	19	16	0.1926
Ly (−)	14	22	
V			
V (+)	6	5	0.5600
V (−)	27	33	

ER, estrogen receptor; HER2, human epidermal growth factor receptor type 2; Ly, lymph node invasion; PgR, progesterone receptor; V, vascular invasion.

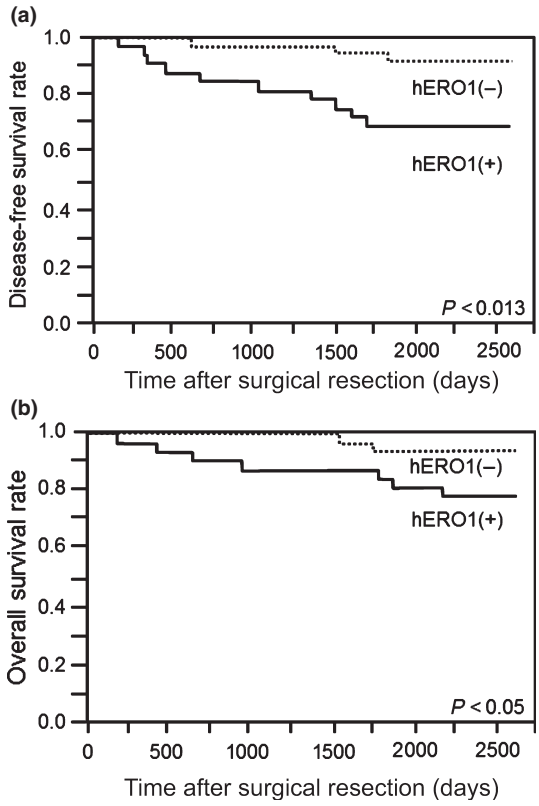

Fig. 7. Kaplan–Meier analysis of disease-free survival (a) and overall survival (b) for human endoplasmic reticulum oxidoreductin 1-α (hERO1-α) expression in 71 cases of invasive breast carcinoma.

hERO1-α, indicating endoplasmic reticulum localization, was found in 33 cases (46.5%) of the 71 patients with breast cancer (Fig. 6c,d). Correlations of hERO1-α (+) and hERO1-α (−) with clinicopathological factors are shown in Table 2. Human ERO1-α (+) type was positively correlated with ER (−) (P = 0.021) and high nuclear grade (P = 0.001). These results suggest that hERO1-α (+) type has a more aggressive phenotype than that of hERO1-α (−) type in breast cancer. No association of hERO1-α (+) type with age, histology, tumor size, or lymph node metastasis was found.

In univariate survival analysis, patients with hERO1-α (+) cancer had significantly shorter disease-free survival (P = 0.01) (Fig. 7a) and overall survival (P = 0.04) (Fig. 7b) than did patients with hERO1-α (−) cancer. In multivariate analysis of disease-free survival by Cox regression analysis, expression of hERO1-α was the only independent prognostic factor (Table 3).

Importantly, we observed the intratumoral heterogeneity of hERO1-α expression, ranging from negative to strong in intensity by immunohistochemistry (Fig. 6c). It has been shown that the hypoxic areas were frequently observed within cancer tissues. As the expression of hERO1-α is induced under hypoxia (Fig. 1d), we assumed that cancer cells residing within hypoxic areas showed augmented expression of hERO1-α. Thus, the heterogeneity of hERO1-α expression seems to be attributed to the oxygen and blood supply. The relationship between hERO1-α and vessel distribution needs to be investigated.

Table 3. Univariate and multivariate survival analyses in breast cancer patients (n = 71)

	Univariate analysis			Multivariate analysis		
	Relative risk	95% CI	*P*-value	Relative risk	95% CI	*P*-value
T (≥2 cm, <2 cm)	0.97	0.30–4.32	0.9602	0.83	0.19–3.34	0.7988
NG (1 + 2, 3)	0.47	0.16–1.47	0.1862	0.80	0.22–3.04	0.7407
LN meta (+, −)	0.87	0.24–2.68	0.8193	0.83	0.19–3.35	0.7988
Ly (+, −)	1.24	0.41–3.85	0.6980	1.44	0.35–5.66	0.6020
V (+, −)	0.44	0.02–2.21	0.3694	0.33	0.02–2.10	0.2640
hERO1-α (+, −)	4.46	1.36–19.90	0.0122	4.13	1.10–20.08	0.0352

CI, confidence interval; hERO1-α, human endoplasmic reticulum oxidoreduction 1-α; LN meta, lymph node metastasis; Ly, lymph node invasion; NG, nuclear grade; V, vascular invasion.

Discussion

In this study, we showed that ERO1-α was overexpressed in the highly metastatic breast cancer cell line 4T1 and in patients with breast cancer recurrence. Depletion of ERO1-α by shRNA in 4T1 cells inhibited *in vivo* tumor growth as well as lung metastasis, suggesting that ERO1-α plays a pivotal role in tumor progression and metastasis. Moreover, a positive correlation was found between hERO1-α expression and recurrence in breast cancer patients, which is mainly caused by dissociation of tumor cells from the primary tumor and dissemination into other sites during tumor progression. Thus, these results suggested that ERO1-α (+) tumor cells were more likely to invade into the stroma and vasculature and then metastasize to remote organs, as indicated by the results of knockdown of ERO1-α. We also found an association between hERO1-α expression and high levels of nuclear grade in clinical specimens, indicating high proliferative activity of tumor cells. Moreover, we showed that ERO1-α plays a pivotal role in VEGF production through disulfide bond formation by PDI within VEGF protein, suggesting that ERO1-α affects tumor growth through angiogenic signaling pathways.[11] Most notably, the fact that the expression of ERO1-α is induced under hypoxic conditions seems to be beneficial to tumor cells to overcome such stressful conditions through production of VEGF-A and other angiogenic factors. These results suggest that ERO1-α plays a pivotal role in survival of cancer cells at the tumor origin against hypoxic conditions, as well as accelerating metastasis through production of angiogenic factors including VEGF-A through disulfide bond formation. In fact, hERO1-α (+) breast cancer patients showed a higher recurrence rate and more dismal outcome after surgery. All of these findings indicate that the expression of hERO1-α is significantly related to aggressive phenotype of breast cancer; hERO1-α can be a new prognostic marker for breast cancer after surgery. However, it should be determined how ERO1-α expression is regulated under hypoxia and within tumor cells and, more importantly, the precise mechanism for tumor cell growth should be elucidated.

Until now, there has been no ideal tumor marker with prognostic value. The predictive significance of hERO1-α in breast cancer could help clinicians identify patients at high risk for recurrence, and enable clinicians to carry out rational adjuvant therapy after surgery. Taken together, our results indicate that hERO1-α may be a suitable prognostic marker for breast cancer.

In addition, the potential for targeting hERO1-α in cancer therapy seems promising, as hERO1-α is overexpressed in many human cancers but is barely detectable in normal tissues. Thus, cancer therapy targeting hERO1-α activity may be a promising strategy for treatment of various types of cancer.

Acknowledgments

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Disclosure Statement

The authors have no conflict of interest.

References

1 Lunt SJ, Chaudary N, Hill RP. The tumor microenvironment and metastatic disease. *Clin Exp Metastasis* 2009; **26**: 19–34.
2 Chaudary N, Hill RP. Hypoxia and metastasis in breast cancer. *Breast Dis* 2006; **26**: 55–64.
3 Milosevic M, Chung P, Parker C *et al.* Androgen withdrawal in patients reduces prostate cancer hypoxia: implications for disease progression and radiation response. *Cancer Res* 2007; **67**: 6022–5.
4 Milosevic M, Warde P, Menard C *et al.* Tumor hypoxia predicts biochemical failure following radiotherapy for clinically localized prostate cancer. *Clin Cancer Res* 2012; **18**: 2108–14.
5 Benham AM, Cabibbo A, Fassio A, Bulleid N, Sitia R, Braakman I. The CXXCXXC motif determines the folding, structure and stability of human ERO1-Lalpha. *EMBO J* 2000; **19**: 4493–502.
6 Gess B, Hofbauer KH, Wenger RH, Lohaus C, Meyer HE, Kurtz A. The cellular oxygen tension regulates expression of the endoplasmic oxidoreductase ERO1-Lalpha. *Eur J Biochem* 2003; **270**: 2228–35.
7 Araki K, Nagata K. Functional in vitro analysis of the ERO1 protein and protein-disulfide isomerase pathway. *J Biol Chem* 2011; **286**: 32705–12.
8 Masui S, Vavassori S, Fagioli C, Sitia R, Inaba K. Molecular bases of cyclic and specific disulfide interchange between human ERO1alpha and PDI. *J Biol Chem* 2011; **286**: 16261–71.
9 Tavender TJ, Bulleid NJ. Molecular mechanisms regulating oxidative activity of the ERO1 family in the endoplasmic reticulum. *Antioxid Redox Signal* 2010; **13**: 1177–87.
10 Tanaka S, Uehara T, Nomura Y. Up-regulation of protein-disulfide isomerase in response to hypoxia/brain ischemia and its protective effect against apoptotic cell death. *J Biol Chem* 2000; **275**: 10388–93.
11 May D, Itin A, Gal O, Kalinski H, Feinstein E, Keshet E. ERO1-L alpha plays a key role in a HIF-1-mediated pathway to improve disulfide bond formation and VEGF secretion under hypoxia: implication for cancer. *Oncogene* 2005; **24**: 1011–20.
12 Qin C, Wilson C, Blancher C, Taylor M, Safe S, Harris AL. Association of ARNT splice variants with estrogen receptor-negative breast cancer, poor induction of vascular endothelial growth factor under hypoxia, and poor prognosis. *Clin Cancer Res* 2001; **7**: 818–23.
13 Schindl M, Schoppmann SF, Samonigg H *et al.* Overexpression of hypoxia-inducible factor 1alpha is associated with an unfavorable prognosis in lymph node-positive breast cancer. *Clin Cancer Res* 2002; **8**: 1831–7.
14 Potgens AJ, Lubsen NH, van Altena MC *et al.* Covalent dimerization of vascular permeability factor/vascular endothelial growth factor is essential for its biological activity. Evidence from Cys to Ser mutations. *J Biol Chem* 1994; **269**: 32879–85.
15 Claffey KP, Senger DR, Spiegelman BM. Structural requirements for dimerization, glycosylation, secretion, and biological function of VPF/VEGF. *Biochim Biophys Acta* 1995; **1246**: 1–9.
16 Muller YA, Li B, Christinger HW, Wells JA, Cunningham BC, de Vos AM. Vascular endothelial growth factor: crystal structure and functional mapping of the kinase domain receptor binding site. *Proc Natl Acad Sci U S A* 1997; **94**: 7192–7.
17 Frand AR, Kaiser CA. ERO1p oxidizes protein disulfide isomerase in a pathway for disulfide bond formation in the endoplasmic reticulum. *Mol Cell* 1999; **4**: 469–77.

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